Imaging, Diagnosis, Prognosis

Effective Assessment of *egfr* Mutation Status in Bronchoalveolar Lavage and Pleural Fluids by Next-Generation Sequencing

Fiamma Buttitta[1,2], Lara Felicioni[1,2], Maela Del Grammastro[1], Giampaolo Filice[1], Alessia Di Lorito[3], Sara Malatesta[1], Patrizia Viola[1], Irene Centi[4], Tommaso D'Antuono[4], Roberta Zappacosta[3], Sandra Rosini[3], Franco Cuccurullo[1], and Antonio Marchetti[1]

Abstract

Purpose: The therapeutic choice for patients with lung adenocarcinoma depends on the presence of EGF receptor (*EGFR*) mutations. In many cases, only cytologic samples are available for molecular diagnosis. Bronchoalveolar lavage (BAL) and pleural fluid, which represent a considerable proportion of cytologic specimens, cannot always be used for molecular testing because of low rate of tumor cells.

Experimental Design: We tested the feasibility of *EGFR* mutation analysis on BAL and pleural fluid samples by next-generation sequencing (NGS), an innovative and extremely sensitive platform. The study was devised to extend the *EGFR* test to those patients who could not get it due to the paucity of biologic material. A series of 830 lung cytology specimens was used to select 48 samples (BAL and pleural fluid) from patients with *EGFR* mutations in resected tumors. These samples included 36 cases with 0.3% to 9% of neoplastic cells (series A) and 12 cases without evidence of tumor (series B). All samples were analyzed by Sanger sequencing and NGS on 454 Roche platform. A mean of $21,130 \pm 2,370$ sequences per sample were obtained by NGS.

Results: In series A, *EGFR* mutations were detected in 16% of cases by Sanger sequencing and in 81% of cases by NGS. Seventy-seven percent of cases found to be negative by Sanger sequencing showed mutations by NGS. In series B, all samples were negative for *EGFR* mutation by Sanger sequencing whereas 42% of them were positive by NGS.

Conclusions: The very sensitive *EGFR*-NGS assay may open up to the possibility of specific treatments for patients otherwise doomed to re-biopsies or nontargeted therapies. *Clin Cancer Res; 19(3); 691–8.* ©2012 AACR.

Introduction

Recent advances in the treatment of neoplastic diseases have been achieved with the introduction of new drugs designed to block molecules and pathways crucial for growth and survival of tumor cells (1, 2). The use of EGF receptor (*EGFR*)-tyrosine kinase inhibitors (TKI), such as gefitinib (Iressa; Astrazeneca) or erlotinib (Tarceva; OSI Pharmaceuticals Inc.), has led to impressive clinical responses in patients with non–small cell lung cancer (NSCLC). Clinical trials have shown that about 75% to 80% of patients with lung cancer carrying *EGFR* mutations attained dramatic radiographic responses to TKI treatment (3). In addition, progression-free survival (PFS) and overall survival (OS) were significantly better for *EGFR*-TKI–treated patients with *EGFR* mutations than those with *EGFR* wild-type. Moreover, *EGFR*-mutated patients treated in the maintenance setting had a large benefit in term of PFS (3, 4). The clinical relevance of *EGFR* mutational status has been deeply discussed in recent years and testing for these mutations has rapidly become a standard practice. A great attention has been focused on the management of biologic samples available for molecular diagnosis (5, 6). Resected tumors are the most informative samples for molecular tests and have been indicated as preferred sample type for *EGFR* analysis (7). Unfortunately, only a minority of patients with NSCLCs, less than 30%, are eligible for surgical resection as advanced staging precludes the surgical approach, thus limiting the availability of neoplastic tissue for molecular analysis (8, 9). About 70% of patients with lung cancer

Authors' Affiliations: [1]Center of Predictive Molecular Medicine, Center of Excellence on Aging; [2]Oncological and Cardiovascular Molecular Medicine Unit, University-Foundation; [3]Department of Biomedical Sciences, University "G. D'Annunzio" of Chieti; and [4]Hospital "SS Annunziata", Pathology Unit, Chieti, Italy

F. Buttitta and A. Marchetti are co-corresponding authors.

F. Buttitta, L. Felicioni, M. Del Grammastro, and G. Filice contributed equally to this work.

Corresponding Author: Antonio Marchetti, Center of Predictive Molecular Medicine, CeSI (Center of Excellence on Aging) University-Foundation, Via Colle dell'Ara, Chieti 66100, Italy. Phone: 39-0871-357407; Fax: 39-0871-540079; E-mail: amarchetti@unich.it

American Association for *Cancer Research*

Translational Relevance

The interest in detecting druggable genetic changes in biologic samples with a very low rate of neoplastic cells is growing. Lung cytology specimens such as bronchoalveolar lavage and pleural fluids may be inadequate for molecular testing because of the very low number of tumor cells in a large excess of normal and inflammatory elements. Over the last decades, the "gold standard" method for gene mutation testing has been Sanger sequencing. However, this technique is not very sensitive, and its application in cytologic specimens is limited. The development of a much more sensitive technology, the massive parallel sequencing, also known as "next-generation sequencing" (NGS), offers new diagnostic opportunities. The *EGFR*-NGS mutation assay used in this study is a very sensitive diagnostic tool potentially useful to avoid re-biopsies or treatment with nontargeted therapies in patients with limited amount of material for molecular diagnosis.

are diagnosed solely by examination of small biopsies and/or cytologic samples (10, 11). Small biopsies are often suitable for dissection and molecular analysis. Cytologic samples cannot always be used for mutational analysis because of paucity of neoplastic cells and/or large cellular heterogeneity, which may require highly sensitive mutation detection methods (12, 13). In particular, bronchoalveolar lavage (BAL) and pleural fluid are less adequate for molecular testing than other biologic specimens achieved by transbronchial or transthoracic fine-needle aspiration (FNA) due to the lower rate of tumor cells (14). In these cases, a possible strategy is to proceed with manual or laser capture microdissection of tumor cells before DNA extraction and molecular analysis. However, these approaches are time-consuming and not suitable for routine diagnostics. In addition, they are error-prone due to a series of problems, including cross-contaminations and artifactual genomic changes that could be encountered while working with low amount of gDNA (15). An alternative strategy is the use of extremely sensitive techniques to detect mutations affecting few tumor cells in a wide excess of non-neoplastic elements.

In the history of genomic sequencing, we are currently experiencing a technical revolution with the advent of next-generation sequencing (NGS; also known as massively parallel sequencing; refs. 16–18). NGS by far exceeds the limits of the traditional Sanger sequencing allowing a huge number of independent sequencing reactions simultaneously. Therefore, this novel approach can allow the detection of genetic mutations even if they are present in a minority of DNA molecules (19). However, the application of high-throughput sequencing in clinical diagnostic setting has been largely unexplored.

We set out to verify the feasibility of an effective assessment of *EGFR* mutations in BAL and pleural fluid specimens by a new sensitive assay based on NGS.

Materials and Methods

Patients and sample characteristics

From a large series of 830 cytologic specimens, obtained from patients with lung adenocarcinoma who underwent surgical resection or biopsy at the Department of Surgery, University of Chieti, during the period February 2005 to March 2011, 95 BAL and pleural fluids corresponding to tissue samples with a documented *EGFR* mutation were selected. All these samples were scanned at high resolution (400× HPF: high-power field), and cells were counted independently by 2 pathologists (F. Buttitta and S. Malatesta) to assess the percentage of neoplastic cells in each sample. Forty-eight cytologic specimens, including 36 cases with less than 10% of neoplastic cells and 12 cases negative for tumor cells, were selected for *EGFR* mutation analysis by deep sequencing. The samples selected included BAL (33 cases) and pleural effusions (15 cases) fixed with methanol, spread on glass slides, and stained with Papanicolaou method for cytologic examination and diagnosis. In addition, 5 cytologic specimens found to be positive for *EGFR* mutations by Sanger sequencing and 10 cytologic samples obtained from patients without *EGFR* mutations in tumor tissue were chosen as positive and negative internal controls, respectively. Informed consent was obtained from all patients included in this study.

DNA extraction and Sanger sequencing

In each case, after removal of the coverslip by immersion in bioclear for 24 to 72 hours, the slide was washed in 95% ethanol for 2 hours, and cells were recovered by scraping the whole content of the sample. No dissection was conducted before DNA extraction that was conducted by the Qiamp DNA Micro kit (Valencia), following manufacturer's instructions. Sanger sequencing analysis of *EGFR* exons 19 and 21 was conducted on the ABI Prism 3100 DNA Analyzer (Applied Biosystem), as previously described (20). Samples harboring mutations were reamplified and resequenced using the same experimental conditions. Sequence chromatograms were analyzed by the Mutation Surveyor 3.0 software (SoftGenetics), followed by manual review.

Next-generation pyrosequencing

After DNA extraction and quantification, the cytologic samples were subjected to PCR amplification and deep sequencing by the 454 GS Junior System (454 Life Sciences and Roche Applied Sciences). The 454 GS Junior technology is derived from the technologic convergence of emulsion PCR and pyrosequencing. Briefly, PCR reactions were carried out by oligonucleotide composite primers (fusion primers), specially designed for *EGFR* exon 19 and 21 (Supplementary Table S1) using the OligoAnalyzer 3.1 software (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primers were synthesized at MWG-Biotech AG. The sequence amplicons were of 206 bp for exon 19 and 189 bp for exon 21. PCR reactions were run in 30 μL reaction volumes, containing 5.5 mmols dNTPs, 11 μmols of each primer, 2.75 μL PCR buffer, 1 μL DNA, and 1.3 units of FastStart HiFi Polymerase (Roche Diagnostics).

Clinical Cancer Research

A touch-down PCR cycling program was conducted on the Gene Amp PCR system 9700 thermocycler (Applied Biosystems) with an initial step at 94°C for 2 minutes followed by 43 cycles at 94°C for 30 seconds, 64°C (decreasing the temperature by 1°C each cycle for 6 cycles) for 30 seconds, and 70°C for 30 seconds, and a final step at 70°C for 5 minutes.

Amplicon products were purified using Ampure SPRI beads (Agencourt Bioscience Corporation), quantified by the Quant-iT PicoGreen Assay (Invitrogen) and then pooled at an equimolar ratio for each individual patient. All samples were processed using the 454 GS Junior Titanium Series Lib-A emPCR Kit (Roche Diagnostics) according to the manufacturer's protocols. Single PCR amplicon molecules were captured on individual 28-μm beads within an oil–water emulsion to enable clonal amplification in a second PCR process with universal primers that yields about 10^7 copies of the input DNA molecule.

The emulsion was then disrupted, the beads were isolated and those with bound emPCR products loaded into a Titanium PicoTiterPlate (Roche Diagnostics) on the 454 GS Junior platform for massively parallel pyrosequencing, according to manufacturer's recommendations.

A number of strategies were adopted to avoid cross-contaminations among different samples and different runs: (i) reactions were set up in positive pressure hoods with UV sterilization systems to decontaminate reagents and equipment before carrying out PCRs; (ii) different hoods were used for PCR amplification of samples subjected to different runs; (iii) PCR reactions were carried out on 96-well plates, with a maximum of 4 samples loaded per plate. In addition, the presence of patient-specific MID sequences within the primers allows the detection of possible cross-contamination products, which are automatically recognized by the software and excluded from analysis.

Analysis of sequence data

Processed and quality-filtered reads were analyzed with the GS Amplicon Variant Analyzer (AVA) software version 2.5.3 (454 Life Sciences). *EGFR* exon 19 and 21 reference sequences were extracted from Hg19 Human Genome Version together with both neighbor intronic regions. Such sequences were used as reference sequences to align every reads. The final alignments were checked manually and wrong alignments were edited by Jalview Multi-alignment Tool (http://www.jalview.org/). To parse and manage all data sequences and results (variations, frequency of mutations, forward and reverse reads check), custom scripts were created using Perl language.

Results

From a series of 95 BAL and pleural fluid specimens, obtained from patients affected by lung adenocarcinomas carrying *EGFR* mutations in tumor tissue, 48 cases were selected for Sanger sequencing and NGS analysis on the basis of the low abundance (<10%) or absence of tumor cells. Of these selected samples, 36 had less than 10% of neoplastic cells, ranging between 0.5% and 9% whereas 12 were judged to be negative for tumor cells. The nonneoplastic component in these samples was composed of normal epithelial cells and inflammatory elements. In most specimens, clusters of necrotic cells and erythrocytes were also present. In each case, the *EGFR* mutation status assessed by Sanger sequencing on the corresponding surgical specimen was considered as a reference to determine the accuracy of molecular tests conducted on cytologic samples. Twenty-two (61%) of the 36 cases positive for tumor cells and 7 (58%) of the 12 morphologically negative specimens showed exon 19 deletions in the matching tumor tissue, the remaining cases showed exon 21 point mutations (Table 1).

EGFR mutation status by Sanger sequencing

The gDNA extracted from the 48 cytologic samples was first subjected to Sanger sequencing that revealed the presence of *EGFR* mutations in only 5 (14%) cases. In particular, Sanger sequencing allowed to detect exon 19 deletions in 4 (18%) of 22 cytologic samples from patients with a documented deletion at exon 19 in matched tumor tissue and a L858R mutation in 1 (7%) of 14 cases carrying exon 21 mutations in tissue samples (Table 1). The percentage of

Table 1. *EGFR* mutational analysis by Sanger sequencing and NGS of cytological samples with and without morphologic evidence of neoplastic cells

EGFR mutational analysis	Surgical samples *EGFR* +ve	Cytological samples *EGFR* +ve	
		Sanger (%)	NGS (%)
With morphologic evidence of neoplastic cells			
Deletion exon 19	22	4 (18%)	19 (86%)
Point Mutation exon 21	14	1 (7%)	10 (71%)
Total number	36	5 (14%)	29 (81%)
Without morphologic evidence of neoplastic cells			
Deletion exon 19	7	0	3 (43%)
Point Mutation exon 21	5	0	2 (40%)
Total number	12	0	5 (42%)

Abbreviations: +ve = positive; NGS = next generation sequencing.

Table 2. *EGFR* mutation analysis on BAL and pleura fluids (Series A) by Sanger sequencing and NGS

| | | | *EGFR* mutational analysis | | |
| | | | Cytology | | Surgical sample |
ID	Sample type	Tumor cells (%)	Sanger sequencing	NGS	Sanger sequencing
1	PF	0.3	Wild-type	Wild-type	2236_2250del 15bp
2	BAL	8	Wild-type	2235_2249del 15bp	2235_2249del 15bp
3	BAL	5	Wild-type	2240_2257del 18bp	2240_2257del 18bp
4	BAL	0.3	Wild-type	Wild-type	T 2573G
5	BAL	9	T 2573G	T 2573G	T 2573G
6	PF	7	2236_2250del 15bp	2236_2250del 15bp	2236_2250del 15bp
7	BAL	5	Wild-type	2235_2249del 15bp	2235_2249del 15bp
8	BAL	0.3	Wild-type	T 2573G	T 2573G
9	BAL	4	Wild-type	2236_2250del 15bp	2236_2250del 15bp
10	PF	7	2235_2249del 15bp	2235_2249del 15bp	2235_2249del 15bp
11	BAL	1	Wild-type	Wild-type	T 2573G
12	BAL	7	Wild-type	2237_2255delinsT 18bp 2237_2257delinsTCT 18bp[a]	2237_2255delinsT 18bp
13	BAL	2	Wild-type	Wild-type	T 2573G
14	PF	1	Wild-type	T2582A	T2582A
15	BAL	9	Wild-type	T 2573G	T 2573G
16	BAL	5	Wild-type	2235_2252delinsATT 15bp 2235_2251delinsAATTC12b[a]	2235_2252delinsATT15bp
17	PF	0.3	Wild-type	Wild-type	2240_2257del 18bp
18	BAL	8	2235_2249del 15bp	2235_2249del 15bp	2235_2249del 15bp
19	PF	7	Wild-type	T 2573G	T 2573G
20	PF	5	Wild-type	T 2573G	T 2573G
21	BAL	0.3	Wild-type	2240_2257del 18bp	2240_2257del 18bp
22	BAL	2	Wild-type	T 2573G	T 2573G
23	BAL	0.3	Wild-type	2235_2249del 15bp	2235_2249del 15bp
24	PF	0.5	Wild-type	2236_2250del 15bp	2236_2250del 15bp
25	BAL	9	Wild-type	T 2573G	T 2573G
26	PF	0.5	Wild-type	Wild-type	2235_2249del 15bp
27	BAL	2	Wild-type	2235_2249del 15bp	2235_2249del 15bp
28	BAL	1	Wild-type	T 2573G	T 2573G
29	PF	5	Wild-type	2235_2249del 15bp	2235_2249del 15bp
30	BAL	0.5	Wild-type	2236_2250del 15bp	2236_2250del 15bp
31	BAL	2	Wild type	2236_2250del 15bp	2236_2250del 15bp
32	BAL	5	2240_2257del 18bp	2240_2257del 18bp	2240_2257del 18bp
33	BAL	0.3	Wild type	Wild type	T 2573G
34	BAL	0.5	Wild type	T 2573G	T 2573G
35	BAL	0.3	Wild type	2237_2255delinsT 18bp	2237_2255delinsT 18bp
36	BAL	0.3	Wild type	2235_2249del 15bp	2235_2249del 15bp

NOTE: Comparison with *EGFR* mutations by Sanger sequencing on matched surgical samples.

Abbreviations: ID, identification number; PF, pleural fluids.

[a]Subclone of DNA molecules carrying a deletion different from the main one.

neoplastic cells was 9% in the cytologic sample with L858R point mutation and ranged between 5% and 8% in the cases with *EGFR* deletions (Table 2). The other 31 samples positive for neoplastic cells and all of the 12 samples without morphologic evidence of malignancy were found to be negative for *EGFR* mutations by Sanger sequencing analysis (Table 1).

EGFR mutational status by NGS

We first evaluated the detection sensitivity of NGS by dilutions experiments. A genomic lung tumor DNA carrying an exon 19 deletion in about 50% of the molecules, as detected by NGS, was progressively diluted (1:10, 1:100, 1:1,000, 1:5,000, 1:10,000, 1:20,000) in wild-type DNA. Each dilution was conducted in 3 replicates. Deep

sequencing of the diluted samples was obtained conducting a mean of 19,870 ± 1,350 sequences per sample (about 20,000×). We were able to detect exon 19 deletions up to a dilution of 1:10,000. Results were comparable in the different replicates, with minimal variations in the percentage of mutated molecules (data not shown).

Mutation analysis of the 48 BAL and pleural fluids by NGS was conducted conducting a mean of 21,130 ± 2,370 sequences per sample. In the series of 36 cytologic samples morphologically positive for neoplastic cells, deep sequencing revealed the presence of *EGFR* mutations in 29 (81%) cases. The percentage of molecules carrying mutations ranged from 0.5% to 15.3% of the gDNA. In particular, the NGS analysis could accurately identify *EGFR* mutations in 24 (77%) of 31 cases judged to be negative by Sanger sequencing (Table 2). All these mutations exactly corresponded to those observed by Sanger sequencing in matching tissue samples. *EGFR* Exon 19 deletions were found in 19 (86%) of 22 cytologic samples from patients with deletions at exon 19 in tumor tissues (Fig. 1). In 2 of these cases (#12 and #16) the NGS showed, in addition to the main deletion, identical to that detected in tumor tissue, the presence of a subclone of DNA molecules carrying a different deletions, structurally related to the main one (see analytic data on Table 3). Exon 21 L858R point mutations were detected in BAL and pleural fluids by NGS in 10 (71%) of 14 cases carrying this type of mutation in tumor samples.

Unexpectedly, in 5 (42%) of the 12 cytologic samples, judged to be negative for neoplastic cells by morphologic examination, the NGS revealed the presence of *EGFR* mutations in 0.3% to 3.2% of the DNA molecules. The types of mutations corresponded exactly to those observed by Sanger sequencing in the matching tissue samples. In these 5 cases, the absence of neoplastic cells in the smears was confirmed by immunohistochemistry (IHC) with anti-TTF1 antibody (DAKO clone 8G7G3/1) on additional smears available (2–3 slides per patient). However, because of the presence of cell conglomerates in these samples, the possibility that the antibody did not react with hidden epitopes cannot be excluded.

Overall, in 7 (19%) of the 36 samples with morphologic evidence of tumor cells and 7 (58%) of the 12 samples judged to be negative for neoplastic cells by morphologic examination, we did not find *EGFR* mutations by NGS. For these 14 cases and positive controls, the PCR-NGS procedure was repeated in the same experimental conditions and results were confirmed (Supplementary Table S2).

The series of 10 negative control samples, obtained from patients without *EGFR* mutations in tumor tissue, was found to be wild-type by Sanger sequencing and NGS.

Discussion

The present study was devised to evaluate a selected series of BAL and pleural fluid specimens for *EGFR* mutations by NGS on the 454 GS Junior platform. All of the BAL and pleural fluid samples selected showed a very low amount of neoplastic cells (less than 10%) or were diagnosed as tumor-free by routine cytology and belonged to patients who had

Figure 1. Example of a deletion at exon 19 of the *EGFR* gene detected by NGS in a BAL specimen (#21) containing a very low amount of neoplastic cells [indicated by a black circle in (A)]. Of the 19,580 sequences obtained in this case, 98 (0.5%) showed a 2240_2257del. Only 19 sequences are shown including 3 sequences carrying the deletion (dotted line; B).

also tumor tissue samples (biopsies and/or resected tumors). The new technical approach used in this study, based on pyrosequencing of emulsion PCR reactions, is one of the most sensitive methods available for the detection of somatic mutations when used in deep sequencing, and its sensitivity depends on the number of sequences obtained per sample (21, 22). In this study, we decided to conduct a deep NGS analysis taking a median of more than 20,000 sequences per sample. In cytologic specimens with low amount of tumor cells, the high sensitivity of deep NGS allowed the detection of *EGFR* mutations in 81% of samples (vs. 14% by Sanger sequencing) and in 77% of the cases that were negative by Sanger sequencing, with a specificity of 100%. Our results indicate that deep NGS is a very specific and sensitive assay that could be useful in clinical practice to assess *EGFR* mutations in BAL and pleural fluid samples.

Cytologic specimens can be obtained with a variety of sampling options including sputum, pleural fluid, bronchoalveolar lavage, bronchial brushing, and, more recently,

Table 3. *EGFR* mutation analysis on BAL and pleura fluids (Series B) by Sanger sequencing and NGS

| | | | EGFR mutational analysis | | |
| | | | Cytology | | Surgical sample |
ID	Sample type	Tumor cells (%)	Sanger sequencing	NGS	Sanger sequencing
1n	BAL	n.a.	Wild-type	Wild-type	2235_2249del 15bp
2n	BAL	n.a.	Wild-type	2235_2249del 15bp	2235_2249del 15bp
3n	BAL	n.a.	Wild-type	2240_2257del 18bp	2240_2257del 18bp
4n	PF	n.a.	Wild-type	Wild-type	T 2573G
5n	BAL	n.a.	Wild-type	T 2573G	T 2573G
6n	PF	n.a.	Wild-type	Wild-type	2236_2250del 15bp
7n	PF	n.a.	Wild-type	Wild-type	2235_2249del 15bp
8n	BAL	n.a.	Wild-type	T 2573G	T 2573G
9n	PF	n.a.	Wild-type	2236_2250del 15bp	2236_2250del 15bp
10n	PF	n.a.	Wild-type	Wild-type	2235_2249del 15bp
11n	BAL	n.a.	Wild-type	Wild-type	T 2573G
12n	BAL	n.a.	Wild-type	Wild-type	T 2573G

NOTE: Comparison with *EGFR* mutations by Sanger sequencing on matched surgical samples.

Abbreviations: ID, identification number; n.a., not assessable; PF, pleural fluids.

transbronchial needle aspiration (TBNA)/endobronchial ultrasound (EBUS) and endoscopic ultrasound (EUS)-guided aspiration. The sampling procedure based on FNA and bronchial brushing can lead to a suitable amount of tumor cells for molecular diagnosis using conventional techniques, although a fair percentage of samples may present a very small amount of tumor cells (23, 10). Cytologic samples obtained by BAL and pleural fluids may show a low percentage of neoplastic cells (14). In many cases, these specimens are judged inadequate for molecular testing because tumor cells, scattered or gathered in very small clusters, cannot easily be dissected from nonneoplastic elements. As these samples could represent the only available material for *EGFR* testing, there is a great need of molecular strategies specifically designed to detect mutations affecting few tumor cells in a large excess of normal cells. Direct sequencing is considered the gold standard for the detection of genetic mutations, but it shows a sensitivity that does not exceed 20% to 25% and therefore, due to the heterozygous nature of mutations, it requires the presence of at least 40% to 50% of cancer cell in the biologic sample (12, 24). As reported in international guidelines for the detection of *EGFR* mutations, the limit of neoplastic cell in biologic samples is fixed to 50% (5). Some authors have reported that direct sequencing can successfully be used to detect *EGFR* mutations in cytologic specimens, but in these studies, cases with low percentage of tumor cells were excluded from the analysis (10, 12).

Nonsequencing-based procedures for mutation detection, more sensitive than Sanger sequencing, such as high-resolution melting (HRM; refs. 25, 26) and restriction fragment length polymorphism (RFLP; ref. 27) have been used on cytologic samples. However, these procedures are screening tests that give indirect evidence of the mutation

status, requiring sequencing to accurately identify the type of mutation. Other PCR-based techniques, such as peptide nucleic acid (PNA)-locked PCR clamping (28), allele-specific quantitative real-time PCR (29), scorpion ARMS (TheraScreen; ref. 30), have been developed for sensitive detection of somatic mutations. The main limitation of these techniques is that they require multiple PCRs and therefore an adequate amount of gDNA, not always available when working with cytologic samples. In addition, they can detect only the mutations for which they have been devised (known mutations; ref. 8) and the sensitivity obtained does not exceed 1:100.

The deep NGS sequencing approach used in this study allowed to reach a sensitivity of 1:10,000 in dilution experiments that means this method is about 100 times more sensitive than therascreen and PNA-LNA clamp and 1,000 times more sensitive than Sanger sequencing. In addition, it can detect any kind of mutation, including novel mutations, requiring minimal amount of gDNA, about 10 ng per PCR reaction. Nowadays, with the advent of the new bench top platforms, such as the 454 GS Junior instrument, there are no main limitations for the application of NGS in clinical practice. The whole process can be accomplished in 2 working days and the cost of reagents per patient is in the order of 200 to 300 Euros if 5 to 6 gDNA samples are examined together.

Interestingly, we observed *EGFR* mutations, exactly corresponding to those detected in tumor tissue, in 42% of the samples without cytopathologic evidence of neoplastic cells. Most of these specimens were particularly dirt, with large and thick cell conglomerates that obscured morphology in many areas. A possible explanation for the results obtained in these cases is that neoplastic cell could have been present in thick conglomerates and therefore not easily

detectable. Alternatively, we could speculate that tumor DNA was present as cell-free DNA or in nuclei debris. This finding further indicates the superior sensitivity of the NSG diagnostic approach and open to other important applications, such as the possibility to use somatic mutations of frequently mutated genes in lung tumors (i.e., *p53*, *KRAS*, *EGFR*) for early diagnosis in fluid samples (sputum, BAL, and pleural effusion) and exhaled breath condensate.

We were unable to detect *EGFR* mutations in 19% of cytologically positive samples analyzed by NGS. This could be ascribed to poor sampling and/or tumor heterogeneity. A number of recent studies indicate that cell heterogeneity is a common finding in different solid tumors, including lung cancer. Because of genetic heterogeneity, in a given tumor, a mutation can be present in a cell clone and not in others, different genes can be mutated in different clones, and the same gene can show different mutations in different clones (31, 32). Particularly noteworthy in this latter respect is the fact that in our series, 2 (6%) cytologic samples showed, in addition to the main mutation corresponding to that observed in matching tumor tissue, subclones of DNA molecules carrying exon 19 deletions different from the main one. The presence of these subpopulations is at the moment unclear. It is extremely unlikely that they are due to cross-contaminations, as they were rare type of *EGFR* deletions, they were confirmed in separate PCRs followed by NGS analysis and they were not seen in control samples. In addition, particular strategies were adopted to minimize cross-contaminations in our study (see Materials and Methods). These subpopulations could represent modifications of the main deletion or *ex novo* deletions acquired in subclones during tumor progression. This finding is in keeping with the hypothesis that lung tumors can be heterogeneous for *EGFR* mutations. We described in detail this novel finding in a dedicated multicentric study conducted on a large series of more than 100 lung tumors carrying *EGFR* deletions at exon 19 (33).

In conclusion, our study indicates that BAL and pleural fluids can be effectively used for the detection of *EGFR* mutations by NGS, even in cases in which conventional methods fail. The high sensitivity and specificity of the deep NGS assay is higher than that reported for other sensitive mutation detection methods. Considering that this new technology is in rapid development, execution times and costs may decrease in the near future, making this new diagnostic approach an ideal method for the assessment of rare mutations in clinical practice.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: F. Buttitta, A. Marchetti
Development of methodology: F. Buttitta, M. Del Grammastro, G. Filice
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Felicioni, A. Di Lorito, S. Malatesta, P. Viola, R. Zappacosta, S. Rosini
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Buttitta, L. Felicioni, M. Del Grammastro, G. Filice, F. Cuccurullo, A. Marchetti
Writing, review, and/or revision of the manuscript: F. Buttitta, A. Marchetti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Del Grammastro, G. Filice, I. Centi, T. D'Antuono, F. Cuccurullo
Study supervision: F. Buttitta, A. Marchetti

References

1. Martini M, Vecchione L, Siena S, Tejpar S, Bardelli A. Targeted therapies: how personal should we go? Nat Rev Clin Oncol 2011; 9:87–97.
2. Gately K, O'Flaherty J, Cappuzzo F, Pirker R, Kerr K, O'Byrne K. The role of the molecular footprint of *EGFR* in tailoring treatment decisions in NSCLC. J Clin Pathol 2012;65:1–7.
3. Mitsudomi T. Erlotinib, gefitinib, or chemotherapy for *EGFR* mutation-positive lung cancer? Lancet Oncol 2011;12:710–1.
4. Milella M, Nuzzo C, Bria E, Sperduti I, Visca P, Buttitta F, et al. *EGFR* molecular profiling in advanced NSCLC: a prospective phase II study in molecularly/clinically selected patients pretreated with chemotherapy. J Thorac Oncol 2012;7:672–80.
5. Pirker R, Herth FJ, Kerr KM, Filipits M, Taron M, Gandara D, et al. Consensus for *EGFR* mutation testing in non-small cell lung cancer: results from a European workshop. J Thorac Oncol 2010;5:1706–13.
6. Marchetti A, Normanno NAIOM-SIAPEC-IAPPinto C, Taddei GL, Adamo V, et al. Recommendations for mutational analysis of *EGFR* in lung carcinoma. Pathologica 2010;102:119–26.
7. Eberhard DA, Giaccone G, Johnson BE. Non-Small-Cell Lung Cancer Working Group. Biomarkers of response to epidermal growth factor receptor inhibitors in Non-Small-Cell Lung Cancer Working Group: standardization for use in the clinical trial setting. J Clin Oncol 2008;26:983–94.
8. da Cunha Santos G, Saieg MA, Geddie W, Leighl N. *EGFR* gene status in cytological samples of nonsmall cell lung carcinoma: controversies and opportunities. Cancer Cytopathol 2011;119:80–91.
9. Travis WD, Rekhtman N, Riley GJ, Geisinger KR, Asamura H, Brambilla E, et al. Pathologic diagnosis of advanced lung cancer based on small biopsies and cytology: a paradigm shift. J Thorac Oncol 2010;5:411–4.
10. Rekhtman N, Brandt SM, Sigel CS, Friedlander MA, Riely GJ, Travis WD, et al. Suitability of thoracic cytology for new therapeutic paradigms in non-small cell lung carcinoma: high accuracy of tumor subtyping and feasibility of *EGFR* and *KRAS* molecular testing. J Thorac Oncol 2011;6:451–8.
11. Malapelle U, Bellevicine C, Zeppa P, Palombini L, Troncone G. Cytology-based gene mutation tests to predict response to anti-epidermal growth factor receptor therapy: a review. Diagn Cytopathol 2011; 39:703–10.
12. Smouse JH, Cibas ES, Jänne PA, Joshi VA, Zou KH, Lindeman NI. *EGFR* mutations are detected comparably in cytologic and surgical pathology specimens of nonsmall cell lung cancer. Cancer 2009; 117:67–72.
13. Hlinkova K, Babál P, Berzinec P, Majer I, Ilencikova D. Rapid and efficient detection of *EGFR* mutations in problematic cytologic specimens by high-resolution melting analysis. Mol Diagn Ther 2011;15: 21–9.

14. Linder J. Lung cancer cytology. Something old, something new. Am J Clin Pathol 2000 114:169–71.
15. Marchetti A, Felicioni L, Buttitta F. Assessing *EGFR* mutations. N Engl J Med 2006;354:526–8; author reply 526–8.
16. Mardis ER. Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet 2008;9:387–402.
17. Schuster SC. Next-generation sequencing transforms today's biology. Nat Methods 2008;5:16–18.
18. Metzker ML. Sequencing technologies-the next generation. Nat Rev Genet 2010;11:31–46.
19. Corless CL, Spellman PT. Tackling formalin-fixed, paraffin-embedded tumor tissue with next-generation sequencing. Cancer Discov 2012;2: 23–4
20. Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, et al. *EGFR* mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 2005;23:857–6
21. Thomas RK, Nickerson E, Simons JF, Jänne PA, Tengs T, Yuza Y, et al. Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. Nat Med 2006;12: 852–55.
22. Querings S, Altmüller J, Ansén S, Zander T, Seidel D, Gabler F, et al. Benchmarking of mutation diagnostics in clinical lung cancer specimens. PLoS One 2011;6:e19601.
23. Billah S, Stewart J, Staerkel G, Chen S, Gong Y, Guo M. *EGFR* and *KRAS* mutations in lung carcinoma: molecular testing by using cytology specimens. Cancer Cytopathol 2011;119:111–7.
24. Thunnissen E, Kerr KM, Herth FJ, Lantuejoul S, Papotti M, Rintoul RC, et al. The challenge of NSCLC diagnosis and predictive analysis on small samples. Practical approach of a working group. Lung Cancer 2012;76:1–18.
25. Nomoto K, Tsuta K, Takano T, Fukui T, Yokozawa K, Sakamoto H, et al. Detection of *EGFR* mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. Am J Clin Pathol 2006;126:608–15.
26. Fassina A, Gazziero A, Zardo D, Corradin M, Aldighieri E, Rossi GP. Detection of *EGFR* and *KRAS* mutations on trans-thoracic needle aspiration of lung nodules by high resolution melting analysis. J Clin Pathol 2009;62:1096–102.
27. Kanaji N, Bandoh S, Ishii T, Kushida Y, Haba R, Kohno K, et al. Detection of epidermal growth factor receptor mutations in a few cancer cells from transbronchial cytologic specimens by reverse transcriptase-polymerase chain reaction. Mol Diagn Ther 2011;15: 353–9.
28. Kawahara A, Azuma K, Sumi A, Taira T, Nakashima K, Aikawa E, et al. Identification of non-small-cell lung cancer with activating *EGFR* mutations in malignant effusion and cerebrospinal fluid: rapid and sensitive detection of exon 19 deletion E746-A750 and exon 21 L858R mutation by immunocytochemistry. Lung Cancer 2011;74: 35–40.
29. van Eijk R, Licht J, Schrumpf M, Talebian Yazdi M, Ruano D, Forte GI, et al. Rapid *KRAS*, *EGFR*, *BRAF* and *PIK3CA* mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. PLoS One 2011;6:e17791.
30. Horiike A, Kimura H, Nishio K, Ohyanagi F, Satoh Y, Okumura S, et al. Detection of epidermal growth factor receptor mutation in transbronchial needle aspirates of non-small cell lung cancer. Chest 2007;131: 1628–34.
31. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
32. Di Lorito A, Schmitt FC. (Cyto)pathology and sequencing: next (or last) generation? Diagn Cytopathol 2012;40:459–61.
33. Marchetti A, Del Grammastro M, Filice G, Felicioni L, Rossi G, Graziano P, et al. Complex mutations and subpopulations of deletions at exon 19 of *EGFR* in lung tumors revealed by new generation sequencing: clinical implications. PLoS One 2012;7:e42164.

AACR American Association for Cancer Research

Clinical Cancer Research

Effective Assessment of *egfr* Mutation Status in Bronchoalveolar Lavage and Pleural Fluids by Next-Generation Sequencing

Fiamma Buttitta, Lara Felicioni, Maela Del Grammastro, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-12-1958
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/12/14/1078-0432.CCR-12-1958.DC1

Cited articles	This article cites 33 articles, 5 of which you can access for free at: http://clincancerres.aacrjournals.org/content/19/3/691.full#ref-list-1
Citing articles	This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/19/3/691.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/19/3/691. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.